Fungi composition in settled dust associated with fractional exhaled nitric oxide in school children with asthma

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HIGHLIGHTS
• We explore the susceptibility of children with asthma to the fungi composition in settled dust.
• The relative abundance of unculturable fungi was associated with FeNO levels.
• PM2.5 exposure was associated with FeNO levels among school children with asthma.
• Unculturable fungi taxa have the potential to exacerbate airway inflammation.

ABSTRACT
Fungi exposure has been significantly linked to respiratory illness. However, numerous fungi taxa that are potentially allergenic still undocumented and leave a barrier to establishing a clear connection between exposure and health risks. This study aimed to evaluate the association of fungi composition in settled dust with fractional exhaled nitric oxide (FeNO) levels among school children with doctor-diagnosed asthma. A cross-sectional study was undertaken among secondary school students in eight schools in the urban area of Hulu Langat, Selangor, Malaysia. A total of 470 school children (aged 14 years old) were randomly selected and their FeNO levels were measured and allergic skin prick tests were conducted. The settled dust samples were collected and analysed by using metagenomic technique to determine the fungi composition. The general linear regression with complex sampling was employed to determine the interrelationship. In total, 2645 fungal operational taxonomic units (OTUs) were characterised from the sequencing process which belongs to Ascomycota (60.7 %), Basidiomycota (36.4 %), Glomeromycota (2.9 %) and Chytridiomycota (0.04 %). The top five mostly abundance in all dust samples were Aspergillus clavatus (27.2 %), followed by Hyphoderma multicystidium (12.2 %), Verrucoconiothyrium prosopidis (9.4 %), Ganoderma tuberculosum (9.2 %), and Heterochaete shearii (7.2 %). The regression results indicated that A. clavatus, Bryocendrichomyces acaciae, C. parapsilosis, H. multicystidium, H. oles, H. shearii, S. melioporinorum, V. prosopidis were associated in increased of FeNO levels among the asthmatic group at 0.992 ppb (95 % CI = 0.34–1.68), 2.887 ppb (95 % CI = 2.09–3.76), 0.809 ppb (95 % CI = 0.14–1.49), 1.757 ppb (95 % CI = 0.59–2.87), 1.088 ppb (95 % CI = 0.51–1.62), respectively. To our knowledge, this is a new finding. The findings pointed out that metagenomics profiling of fungi could enhance our understanding of a complex interrelation between rare and unculturable fungi with airway inflammation.

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1. Introduction

The importance of an indoor microenvironment in school settings for children’s health is widely recognised. Many reviews have established a relationship between indoor school exposure and respiratory morbidities in school children (Chithra and Nagendra, 2018; Esty et al., 2019). Experimental and epidemiological studies have also shown that exposure to the compositions of indoor pollutants such as nitrogen dioxide (NO₂), carbon monoxide (CO), ozone (O₃), volatile organic compounds (VOCs), polycyclic aromatic hydrocarbons (PAHs), and particulate matter is associated persistently with increased asthma exacerbation and aggravation of allergic reaction (Khreis et al., 2017; Lee et al., 2014). Apart from these indoor pollutants, aeroallergens exposure has attracted great attention in the last several years, particularly fungi. A substantial number of reports have demonstrated that several fungal genera such as Aspergillus, Penicillium, Alternaria and Cladosporium might be influenced by the indoor air pollutants and may be associated with airway inflammation and worsening of asthma (Liu et al., 2019; Liu et al., 2018a; Ziska, 2020). However, there is paucity research on the evaluation of unculturable fungi in school settings on airway inflammation response. Also, the health effects of indoor fungi exposure on adolescents and adults are ambiguous (Fu et al., 2020). Furthermore, indoor fungi composition does seem to be geographically, seasonally, temperature and latitude patterned (Banchi et al., 2020; Chen et al., 2019). Review has described that indoor fungi diversity is unevenly reported around the world, with European regions receiving the most attention, while tropical regions are relatively less well documented (Anees-Hill et al., 2022). Overall, more fungi-health association studies encompassing various geographical areas, age groups and indoor settings are required.

In recent years, the characterisation of fungal diversity in different niches using next generation sequencing (NGS) and metagenomics analysis has received tremendous attention. These robust techniques have become more convenient tools for assessing the fungal pathogen and distribution patterns compared to culture-independent methods (Behzad et al., 2015; Yang et al., 2018). More recently, Fu et al. (2021) successfully characterised 744 fungal species using metagenomics profiling in dust samples collected from classrooms in the Province of Shaxi, China. They found that the most genera sequenced were Alternaria, Aspergillus and Mucor. This is in contrast with our previous findings that Aspergillus, Penicillium and Cladosporium were dominated in settled dust samples in schools in Johor Baharu, Malaysia (Fu et al., 2020). As the literature on metagenomics application in fungal study is still growing, the information about allergenic fungi and unculturable genera are very limited.

Respiratory allergic diseases such as asthma, allergic rhinitis and allergic rhinoconjunctivitis are common diseases, with a major impact on quality of life characterised by inflammation, hyperresponsiveness, and reversible obstruction of airways (Beggs, 2010). Hence, great strides have been made in the discovery, application and implementation of biomarkers to comprehend diagnosis, evaluate inflammation in the airways, evaluate hyperresponsiveness for certain measurements of lung function and monitor the activity and prognostic in the treatment process (James and Hedlin, 2016). Nitric oxide (NO) is the most widely used biomarker and found to be directly correlated with the severity of airway inflammation (Kamaruddin et al., 2019; Karrasch et al., 2017). NO is endogenously generated through the oxidation of amino acid L-arginine by neural NOS (nNOS), endothelial NOS (εNOS), and inducible NOS (iNOS) that are expressed in several cells such as epithelial cells, inflammatory cells (macrophages, neutrophils, and mast cells), airway nerves, and vascular endothelial cells (Kim et al., 2016). To date, several previous studies on NGS profiling of fungi have provided initial evidence that only a few fungi genera such as Aspergillus fumigatus, Aspergillus versicolor, Penicillium, Aspergillus/ Penicillium DNA, Candida albicans were associated significantly with FeNO levels (Norbäck et al., 2017b; Rick et al., 2020). However, many other fungi genera may be potentially attributable to the risk of airway inflammation are largely unexplored.

Therefore, the present study aimed to evaluate the associations of fungi composition in settled dust with FeNO levels in a cohort of school-age doctor-diagnosed asthma and non-asthmatic children. We hypothesized that exposure to unculturable fungi extracted from settled dust in a school environment was associated with increased airway inflammation.

2. Methodology

2.1. Study population

A total of 470 school children aged 14 from eight randomly selected secondary schools in the Hulu Langat district, Selangor, Malaysia were enrolled in this cross-sectional study. Hulu Langat district features an urban sprawl from the rapid urbanisation of Kuala Lumpur and Putrajaya, which has massive construction development projects for the industrial and property estates (Bakeri et al., 2020). They were randomly selected from four classrooms in each school. In the selection process, school children who started attending the same school since January 2017 (or >18 months) and obtained written consent from parents or legal guardians with the addition of their own assent were included. On the other hand, school children with concomitant heart diseases and severe asthma conditions were excluded. For the purpose of this study, school children at age of 14 were selected due to specific reasons. First, asthma prevalence in Malaysian children aged 13–14 years increased significantly, with a percentage prevalence change from 5.8 % to 8.9 % for children aged 6–7 and 13–14 years old, respectively (Manan et al., 2017; Md.Sharif et al., 2019). Furthermore, the overall lifetime prevalence of asthma (asthma ever) was reported worldwide increased by 0.28 % per year in the 13–14 year age group (Asher et al., 2020). Second, the evidence about the association between indoor pollutants and asthma morbidity outcomes for this age group has been inadequate, especially for certain fungi taxa. Lastly, these 14 years-old school children have been attending the same school since January 2017, which indicates that they have been exposed to the same school microenvironment for a year.

Sample size calculation was adjusted for stratification sampling using a design effect of 1.1. Due to the scarcity of information from previous studies on the comparison of asthmatic and non-asthmatic at local settings, 0.02 was considered as an anticipated value for inter-cluster correlation (ICC) based on a comparable study conducted in children (Liaw et al., 2008).

The clinical assessment and indoor air monitoring were carried out at the same time from August until November 2018 and in early February 2019. This study was approved by the Ethics Committee for Research Involving Human Subjects Universiti Putra Malaysia (JKEUPM) (JKEUPM-2018-189).

2.2. Clinical assessment

Information on demographic characteristics, doctor’s diagnosed asthma, duration of asthma, any asthma attack during the last 12 months and airway infection for the last 3 months were collected by self-administrative questionnaire. The questionnaire was adapted from the International Study of Asthma and Allergies in Childhood (ISAAC), the European Community Respiratory Health Survey (ECRHS) and previous studies (Cai et al., 2011; Ma’pol et al., 2019; Norbäck et al., 2017a). This information was verified during face-to-face interviews and telephone calls with the children’s respective guardians.

Airway inflammation was assessed by measuring the fractional exhaled nitric oxide (FeNO) using chemiluminescence analyser (NIOX VERO, Circassia, Sweden) with a detection limit and accuracy of 5–300 ppb and ±5 ppb, respectively. This analyser has visual and audio signals that guide the school children to achieve the desired inspiratory flow of 50 mL·s⁻¹ in 6 to 10 s. Samples of exhaled breath were taken in accordance with the standard and as recommended by the manufacturer (Dweik et al., 2011). A single-breath technique was used and this procedure was repeated at least twice to get an average value. School children were instructed to avoid eating and drinking for at least an hour before the FeNO assessment. To exclude errors related to the sampling time, all the FeNO assessment was performed in the afternoon by trained enumerators.
An allergy skin prick test (SPT) was performed using a lancet and five common allergens supplied by ALK-Abellô, (Madrid, Spain): Dermatophagoides pteronyssinus (Derp1) (house dust mite), Dermatophagoides farinae (Derf1) (house dust mite), Cladosporium herbariun (fungi), Alternaria alternata (fungi), and Felis domesticus (cat). Histamine (10 mg/mL) and glycerol-saline were used as the positive and negative controls, respectively. The allergy skin prick test procedures were performed on the volar side of the forearm by trained medical assistants and strictly according to the Australasian Society of Clinical Immunology and Allergy guidelines (ASCIA, 2016). The allergy SPT results were reported 15 min afterwards as the mean diameter of wheal reaction. A wheal reaction of >3 mm was considered as a positive result. Atopy was defined as a positive SPT test response for at least one of the applied allergens (ASCIA, 2016).

2.3. Assessment of school environment

Four classrooms in each school were randomly selected and inspected for signs of dampness or mould growth. Indoor temperature (°C), relative humidity (%), and CO₂ (ppm) were monitored in the classrooms within an hour by using a Q-TrakTM IAQ monitor (Model 7565 TSI Incorporated, Shoreview, Minnesota, USA) with the average log interval values over 1 min. The concentrations of PM₁₀ and PM₂.₅ were measured using two separate units of Dust-Trak monitor (Model 8532 TSI Incorporated, Shoreview, Minnesota, USA) at a sampling rate of 1.7 L/min. All of these samplers were always placed 1 m above floor level and 1 m away from the school children in the centre of the classrooms.

In each school, a total of 4 h of measurements were collected for all of these parameters during the learning session and have been previously described (Kamaruddin et al., 2016; Norbäck et al., 2014; Suhaïmi et al., 2017). For measurement of NO₂, the IVL diffusion samplers (IVL, Goteborg, Sweden) were used with the limit of detection (LOD) of 0.5 µg/m³ and 10.0 % of measurement uncertainty (Foldvary et al., 2017). This sampler was used to determine the average concentration of NO₂ in the air for a week. All the monitoring devices were sent for factory calibration periodically. In particular, the DustTrak Airborne Particle monitors were calibrated zero offset regularly to prevent artefact jumps effects (Rivas et al., 2017).

The settled dust in the classrooms were sampled using an 1800 W vacuum cleaner (Model PVC-31A, Penson, Penang, Malaysia) equipped with an external dust Millipore Filter kit (ALK Abello, Horsholm, Denmark). The dust filter with a pore size of 6 µm was attached to the filter holder (ALK Abello, Horsholm, Denmark) and fitted at the end of vacuum’s tube. The settled dust was collected for a period of 4 min for each sample; 2 min on the floor and another 2 min on the surfaces of desks, chairs, bookshelves and soft boards. The classroom was divided into the window zone (part A) and the corridor zone (part B), which were sampled separately as in previous studies (Fu et al., 2020; Norbäck et al., 2016a; Norbäck et al., 2016b; Norbäck et al., 2017b). In total, 64 settled dust samples were collected from eight schools and they were kept in an individual sterile zip lock plastic bag, and stored in a low freezer at ~80 °C until extraction procedures (Norbäck et al., 2014).

2.4. DNA extraction and amplicon sequencing analysis

The settled dust samples from part A and part B were combined and sieved through a 0.3 mm mesh screen prior extraction process (Norbäck et al., 2014). The fungal genome DNA was extracted from 1.0 g of sieved settled dust by using the EZ-10 Column Soil DNA Mini Prep Kit (BioBasic, Markham, Canada). The manufacturer’s instructions were followed, with modification at the disruption step by 1.0 g addition of sterile 0.6 mm diameter glass beads (Sigma, St. Louis, MO) (Rodrigues et al., 2018). The purity and concentration of DNA extraction were estimated by using NanoPhotometer P-Class (P360) (IMPLEN, Schatzbogen, Germany). The DNA purity and concentration of all samples were between 1.8 and 2.0 and 25–816 ng/μL, respectively. The final products of fungal genome DNA were stored at ~20 °C before further downstream analysis.

Further, the extracted DNA was amplified using primers pair of ITS1–30 F/ITS1–217R (Usyk et al., 2017) with a partial Illumina Nextera adapter incorporated their 5’ end targeting the fungal internal transcribed spacer (ITS) 1 region. The PCR reaction was performed using YourTaq™ Direct-Load PCR Mix (Biotechnabbit, Hennigsdorf, Germany) supplemented with 2.5 % DMSO, with the following protocol: initial denaturation at 95 °C for 180 s, followed by 30 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s and extension step at 72 °C for 30 s. The amplified PCR products were pooled, bead-purified and quantified using Denovix high-sensitivity fluorescence quantification kit (Denovix, Delaware, USA). Paired-end sequencing was further analysed on an ISeq100 (Illumina, San Diego, CA) using the run configuration of 1 × 300 bp to maximise information on the Read 1. Sequencing protocol was performed at certified laboratory GeneEQ, Rawang, Selangor Malaysia.

The fastq files generated by Illumina sequencing were trimmed with Cutadapt v1.18 (Martin, 2011) and the QIIME2 Classifier with UNITE database (version 04.02.2020) (Nilsson et al., 2019) pipelines were used to process the raw sequencing read. Finally, MicrobiomeAnalyst, a web-based platform was used to generate taxa abundance plots, rarefaction curves and alpha diversity from QIIME2. More extensive information on fungal genome DNA extraction and bioinformatics sequence analysis could be found in our previous publication (Mohd Isa et al., 2021).

2.5. Statistical analysis

Descriptive test analysis was performed by the Statistical Package for Social Science (SPSS) 25.0. Levels of FeNO were log-transformed to improve distribution normality in the regression analysis. The Chi-square test was used to compare the school children’s characteristics between doctor-diagnosed asthma and non-asthmatic group, while Mann-Whitney test for weight, height and FeNO levels. Association between the relative abundance of fungi, indoor parameters and FeNO levels were examined by general linear regression (GLR) model with complex sampling controlling for atopy, gender, duration of asthma, asthma attack in the past 12 months, airway infection in the past 3 months, body mass index (BMI), parental asthma/allergy and family member smoking status (Gebremariam et al., 2017; Horvath et al., 2005; Pignatti et al., 2020). The analyses were adjusted with complex sampling of double-stage cluster, school and classroom levels. To be cautious about generalizing the findings, we determine the associations separately for all school children (model 1) and each group (doctor-diagnosed asthma (model 2) and non-asthma (model 3)). The models reasonably fit well, met the model assumptions and there was no significant interaction between independent variables, and no multicollinearity problem identified. Additionally, we incorporated the sampling weights (Foy, 1997) in the multivariate analysis stage to compensate for the unequal probability of selection at classroom and school children levels (Hahs-Vaughn, 2005). All tests were 2-tailed, and a p-value of <0.05 was considered significant.

3. Results

3.1. Descriptive statistics

Table 1 summarised the personal characteristics of school children involved in this study. About 288 (61.3 %) of school children were female. The majority of them were Malay with a percentage of 86.8 %. Information gathered from the self-administered questionnaire set revealed that only 50 (10.6 %) school children were claimed to have been diagnosed with asthma by a medical doctor, and among those, 94.7 % experienced an asthma attack at any time in the past 12 months. The median duration of asthma since diagnosis for doctor-diagnosed asthma group was 8.0 years (IQR = 6.3). Airway infection was more commonly reported by non-asthmatic school children (84.6 %). The allergy skin prick test revealed that about 271 (57.7 %) school children were positive for at least one of the allergens tested. Furthermore, among the doctor-diagnosed asthma group, 14.4 % of them were classified as atopic.
A total of 17.4 % of the school children with asthma had parents with allergies and/or asthma. The prevalence of current smoking was 6.4 %. Approximately 60.6 % of school children were more likely to be exposed to secondhand smoke (SHS) at home. The average FeNO levels in the doctor-diagnosed asthma group (median = 56 ppb, IQR = 63) were statistically higher than in the non-asthmatic group (median = 20 ppb, IQR = 23) (p < 0.001).

### 3.2. Classroom inspection and indoor environmental parameters

Generally, all classrooms were equipped with three ceiling fans, naturally ventilated and designed with glass jalousie window panes on both sides of the wall. The floor surface was finished with concrete. About 25 (78.1 %) classrooms had the sign of dampness or mould growth. About 12.5 % classrooms were used wooden chairs while the other used plastic chairs. There were bookshelves, whiteboards, and soft boards in every classroom.

**Table 2** tabulated the indoor air measurement inside the classrooms.

The ranges of temperature and relative humidity were 27 °C–32 °C and 63.6 %–88.1 %, respectively. The median value of indoor CO₂ was 454 ppm (IQR = 35). The average concentration of NO₂ for a week was 30.0 μg/m³ (IQR = 18) recorded for 4 h measurement ranged between 32.3 – 48.0 μg/m³ and 17.9–26.9 μg/m³, respectively.

### 3.3. Diversity of fungi inside the classrooms

Based on 97 % nucleotide sequence similarity, the clustering sequences recovered 1869 unique operational taxonomic units (OTUs) from all samples. However, only 82 (74.5 %) taxa were identified at the family level.

**Table 1** FeNO levels and characteristics of school children participating in this study.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Overall (n = 470)</th>
<th>Doctor-diagnosed asthma (n = 50)</th>
<th>Non-asthma (n = 420)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>182 (38.7)</td>
<td>27 (14.8)</td>
<td>155 (85.2)</td>
<td>0.019*</td>
</tr>
<tr>
<td>Female</td>
<td>288 (61.3)</td>
<td>23 (8.0)</td>
<td>265 (92.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malay</td>
<td>408 (86.8)</td>
<td>44 (10.8)</td>
<td>364 (89.2)</td>
<td>0.792</td>
</tr>
<tr>
<td>Non-Malay</td>
<td>62 (13.2)</td>
<td>6 (9.7)</td>
<td>56 (90.3)</td>
<td></td>
</tr>
<tr>
<td><strong>Atopic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>271 (57.7)</td>
<td>39 (14.4)</td>
<td>232 (85.6)</td>
<td>0.002*</td>
</tr>
<tr>
<td>No</td>
<td>199 (42.3)</td>
<td>11 (5.5)</td>
<td>188 (94.5)</td>
<td></td>
</tr>
<tr>
<td><strong>Parental allergy/asthma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>155 (33.0)</td>
<td>27 (17.4)</td>
<td>128 (82.6)</td>
<td>0.001*</td>
</tr>
<tr>
<td>No</td>
<td>315 (67.0)</td>
<td>23 (7.3)</td>
<td>292 (92.7)</td>
<td></td>
</tr>
<tr>
<td><strong>Smoking</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>30 (6.4)</td>
<td>6 (20.0)</td>
<td>24 (80.0)</td>
<td>0.158</td>
</tr>
<tr>
<td>No</td>
<td>440 (93.6)</td>
<td>44 (10.0)</td>
<td>396 (90.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Parental/sibling smoking</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>285 (60.6)</td>
<td>37 (13.0)</td>
<td>248 (87.0)</td>
<td>0.041*</td>
</tr>
<tr>
<td>No</td>
<td>185 (39.4)</td>
<td>13 (7.0)</td>
<td>172 (93.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Airway infection in the past 3 months</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>91 (19.4)</td>
<td>14 (15.4)</td>
<td>77 (84.6)</td>
<td>0.102</td>
</tr>
<tr>
<td>No</td>
<td>379 (80.6)</td>
<td>36 (9.5)</td>
<td>343 (90.5)</td>
<td></td>
</tr>
<tr>
<td><strong>Asthma attack in the past 12 months</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>19 (4.0)</td>
<td>19 (4.0)</td>
<td>1 (5.3)</td>
<td>&lt;0.001 **</td>
</tr>
<tr>
<td>No</td>
<td>451 (96.0)</td>
<td>32 (7.1)</td>
<td>419 (92.9)</td>
<td></td>
</tr>
<tr>
<td><strong>Duration of asthma (year)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>8.0 (6.3)</td>
<td>0 (0)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td><strong>FeNO levels (ppb)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>21 (27)</td>
<td>56 (63)</td>
<td>20 (23)</td>
<td>&lt;0.001 **</td>
</tr>
<tr>
<td>No</td>
<td>46 (18)</td>
<td>50.5 (19.8)</td>
<td>46.0 (18.0)</td>
<td>0.241</td>
</tr>
<tr>
<td><strong>Height (m)</strong></td>
<td>1.57 (0.09)</td>
<td>1.58 (0.12)</td>
<td>1.57 (0.09)</td>
<td>0.014*</td>
</tr>
</tbody>
</table>

Values are the median (IQR) for duration of asthma, FeNO levels, weight, and height. IQR = interquartile range. NA = Not available.

* p < 0.05
** p < 0.001

### 3.4. The relative abundance of fungi taxa associated with FeNO levels

The initial analysis model that considered all school children shows that only the relative abundance of fungi taxa was found to be significant predictors of increased FeNO levels (p < 0.05). The fungi taxa of *B. acaciae, H. aloes, T. pseudoveoloxum,* and *V. prosopidis* which belong to phylum Ascomycota as well as *H. multicystidium* and *H. shearii* from phylum Basidimycota were explained about 27.6 % (R² = 0.276) of the variability of FeNO levels in this model (Table 3).

Based on the doctor-diagnosed asthma subgroup analysis, there were eight taxa of fungi significantly associated with the elevation of FeNO levels (p < 0.05), including *A. clavatus, B. acaciae, C. parapsilosis, H. aloes, H. multicystidium, H. shearii, S. meliponinorum,* and *V. prosopidis.* Hence, one unit change of *A. clavatus, B. acaciae, C. parapsilosis, H. aloes, H. multicystidium, H. shearii, S. meliponinorum,* and *V. prosopidis* result in increased of FeNO levels at 0.992 ppb (95 % CI = 0.29 – 1.68), 2.887 ppb (95 % CI = 2.09 – 3.76), 0.809 ppb (95 % CI = 0.14 – 1.49), 0.647 ppb (95 % CI = 0.36 – 0.94), 1.442 ppb (95 % CI = 0.29 – 2.61), 1.757 ppb (95 % CI = 0.59 – 2.87), 1.092 ppb (95 % CI = 0.43 – 1.75) and 1.088 ppb (95 % CI = 0.51 – 1.62), respectively. The concentration of PM₂.₅ was also found to be a significant predictor with one unit change resulting in elevation of 0.269 ppb of FeNO levels (95 % CI = 0.04 – 0.61). Overall, this model explained 87.2 % (R² = 0.872) of the variability of FeNO levels among doctor-diagnosed asthma group.

**Table 2** Average and range concentration of physical parameters and pollutants inside the classrooms.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Median (IQR)</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>28.0 (1.0)</td>
<td>27.9</td>
<td>32.0</td>
<td>23 – 26°C</td>
</tr>
<tr>
<td>Relative humidity (%)</td>
<td>79.2 (14.3)</td>
<td>63.6</td>
<td>88.1</td>
<td>40 – 70%</td>
</tr>
<tr>
<td>PM₁₀ (μg/m³)</td>
<td>37.6 (8.5)</td>
<td>32.3</td>
<td>48.0</td>
<td>50₁0, 15₀, 12₀</td>
</tr>
<tr>
<td>PM₂.₅ (μg/m³)</td>
<td>23.7 (3.0)</td>
<td>17.9</td>
<td>26.9</td>
<td>25₉, 35₉, 50₉</td>
</tr>
<tr>
<td>CO₂ (ppm)</td>
<td>454.0 (35.0)</td>
<td>402.0</td>
<td>471.0</td>
<td>&lt;1000₉₉,₈₀</td>
</tr>
<tr>
<td>NO₂ (μg/m³)</td>
<td>30.0 (18.0)</td>
<td>15.0</td>
<td>48.0</td>
<td>20₀, 10₀ ppb, 7₅</td>
</tr>
</tbody>
</table>

N = 32 of sampling locations/classrooms.

IQR = Interquartile range.

* a Industrial Code of Practice on Indoor Air Quality (ICOP-IAQ) 2010.
* b World Health Organization (WHO) guideline.
* c The National Ambient Air Quality Standard by USEPA.
* d The new Malaysian Ambient Air Quality Standard 2018 Interim Target-2.
Interestingly, the relative abundance of *B. acaciae*, *H. aloes*, *H. multicystidium*, *H. shearii* and *T. pseudoveloxum* were also significantly associated with elevation of FeNO levels among non-asthma group \((p < 0.05)\). All these fungi taxa explained about 24.1 \%(R^2 = 0.241)\) of the variability of FeNO levels among non-asthma group.

Overall, only the concentrations of PM\(_{2.5}\) and the relative abundance of fungi taxa were significantly associated with the elevation of FeNO levels among doctor-diagnosed asthma subgroup. Moreover, the analysis revealed that the relative abundance of *B. acaciae*, *H. aloes*, *H. multicystidium* and *H. shearii* were significantly associated with FeNO levels in all three models.

**Fig. 1.** Relative abundance of fungal identified at the species level in settled dust collected in 32 classrooms.

**Fig. 2.** Different alpha diversity of eight schools were analysed using (A) observed OTU richness, (B) Chao1, and (C) Shannon index.
4. Discussion

This cross-sectional study explored the association of FeNO levels in 470 school children with the relative abundance of fungi characterised in the settled dust collected in the school environment. We successfully sequenced and characterised 109 of fungi taxa in the settled dust samples. Our findings demonstrated that the relative abundance of eight fungi species and concentration of PM$_{2.5}$ were significantly associated with increased FeNO levels among doctor-diagnosed asthma group. To the best of our knowledge, this is the first epidemiological study to explore the inter-relation of unculturable and rare fungi taxa with FeNO levels. Previous studies just focused on specific fungi taxa such as Aspergillus versicolor, Septomyces sp. Alternaria and Cladosporium (Norbäck et al., 2016a; Tham et al., 2019).

In this current study, we found that the prevalence of doctor-diagnosed asthma and atopy was 10.6 % and 57.7 %, respectively. On the other hand, the latest prevalence of doctor-diagnosed asthma among children aged 13 to 14 reported from local studies in Terengganu, Malaysia and Penang, Malaysia was 8.4 % (Ma'pol et al., 2019) and 10.3 % (Norbäck et al., 2017a), respectively. Compared to other studies in South East Asia, the prevalence of doctor-diagnosed asthma in Bangkok, Thailand, Singapore and Surabaya, Indonesia was 8.8 % (Chiraratapanisit et al., 2019), 10.0 % (Goh et al., 2021) and 6.8 % (Soegiarto et al., 2019), respectively. Therefore, there seems to be an indication that asthma prevalence is increasing in Malaysia. Overall, in most countries, an increased prevalence of asthma has been documented compared to the past century (Lundbäck et al., 2016). Moreover, according to Sembajwe et al. (2010), the prevalence of doctor-diagnosed asthma across the world regions was reflected by the national incomes. Likewise, the prevalence of atopy identified by previous studies also shows lower percentages than in this current study. Previous studies in Terengganu, Malaysia, Surabaya Indonesia and Korea found that the prevalence of atopy among similar age group were 40.3 % (Ma’pol et al., 2019), 29.0 % (Soegiarto et al., 2019) and 27.3 % (Park et al., 2016), respectively. Furthermore, this study provided additional epidemiological evidence regarding the higher FeNO levels among asthmatic school children than non-asthmatic with the average value above the threshold of 35 ppb, which could reflect a higher degree of airway inflammation (Dweik et al., 2011). Similar pattern was also observed in previous studies conducted in Peninsular Malaysia (Ma’pol et al., 2019; Norbäck et al., 2017a; Norbäck et al., 2017b) and China (Xu et al., 2011).

Overall, we found that the concentration of PM$_{10}$, PM$_{2.5}$, NO$_2$, CO$_2$ measured inside the classrooms were below the guideline limits set by the World Health Organisation (WHO) guideline (WHO, 2018), the National Ambient Air Quality Standard by USEPA (Environmental Protection Agency, 2021), the new Malaysian Ambient Air Quality Standard 2018 Interim Target-2 (Department of Environment Malaysia, 2021) and American Society of Heating, Refrigerating and Air-Conditioning Engineers.

Table 3
Factors influencing the FeNO levels among school children.

<table>
<thead>
<tr>
<th>Group/explanatory variable</th>
<th>Antilog β</th>
<th>95 % CI</th>
<th>R²</th>
<th>Model 1 (N = 470)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>0.68</td>
<td>(−6.03-4.85)</td>
<td>0.276</td>
<td>Model 1</td>
</tr>
<tr>
<td>B. acaciae</td>
<td>0.367</td>
<td>(0.05-0.74)</td>
<td>Model 2 (n = 50)</td>
<td></td>
</tr>
<tr>
<td>H. aloes</td>
<td>0.181</td>
<td>(0.08-0.31)</td>
<td>0.872</td>
<td>Doctor-diagnosed asthma (n = 50)</td>
</tr>
<tr>
<td>H. multicystidum</td>
<td>0.142</td>
<td>(0.02-0.26)</td>
<td>0.490</td>
<td>Non-asthma (n = 420)</td>
</tr>
<tr>
<td>H. shearii</td>
<td>0.202</td>
<td>(0.06-0.53)</td>
<td>0.31</td>
<td>Intercept</td>
</tr>
<tr>
<td>V. prosopidis</td>
<td>0.164</td>
<td>(0.03-0.35)</td>
<td>0.74</td>
<td>PM$_{2.5}$</td>
</tr>
<tr>
<td>P. clavatus</td>
<td>0.992</td>
<td>(0.34-1.68)</td>
<td>0.26</td>
<td>B. acaciae</td>
</tr>
<tr>
<td>P. parapsilosis</td>
<td>0.809</td>
<td>(0.14-1.49)</td>
<td>0.24</td>
<td>H. aloes</td>
</tr>
<tr>
<td>P. clavatus</td>
<td>1.442</td>
<td>(0.29-2.61)</td>
<td>0.24</td>
<td>H. multicystidum</td>
</tr>
<tr>
<td>V. prosopidis</td>
<td>1.092</td>
<td>(0.43-1.75)</td>
<td>0.24</td>
<td>H. shearii</td>
</tr>
<tr>
<td>V. prosopidis</td>
<td>0.35</td>
<td>(0.24-0.49)</td>
<td>0.24</td>
<td>PM$_{2.5}$</td>
</tr>
</tbody>
</table>

CI = Confidence interval, R² = Coefficient of determination.
* p < 0.05.
** p < 0.001.

Fig. 3. A PCoA plot of Bray-Curtis index based for eight schools and validated with Permutational MANOVA. F = 2.267, R² = 0.398, p < 0.001.
et al., 2020; Xu et al., 2020). Moreover, an in vitro study revealed that a study conducted by Salam et al. (2012) uncovered an interaction between DNA methylation variation within NOS2, epigenetic variations and short-term PM2.5 exposure jointly affecting the FeNO levels in children. However, exposure to PM2.5, NO2 and O3 was not statistically associated with NOS2 methylation. This finding would suggest that further research with the application of genome approach to identify the air pollutants-associated epigenetic variations using longitudinal study designs is needed (Ji et al., 2016).

An important finding in this present study was the significant association of the unculturable fungal identified in settled dust samples with increased FeNO levels among school children with asthma and non-asthma conditions. The relative abundance of four taxa, namely B. acaciæ, H. does, H. multicystidium and H. sheari were generally abundant and significantly associated with FeNO levels in both groups. Nevertheless, no further explanation can be made from this result as these species and their respective genus were not commonly reported in relation to the respiratory illness and generation of NO pathways. Numerous potentially allergenic fungi taxa are still undocumented and leave a barrier to establishing a clear connection between respiratory problems and fungi exposure (Mbareche et al., 2020). Moreover, rare fungi have newly been implicated in human infection due to the continued expansion of investigative technology, human behaviours and environmental factors (Liu et al., 2018b). To the researchers' knowledge, this study is the first study that explores the interaction of several unculturable fungi with FeNO levels among school children.

Several studies have remarked the association of A. clavatus with invasive aspergillosis, extrinsic alveolitis, endocarditis, mycotoxicoses-related diseases and neurologic disorders (Aquino et al., 2018; Richardson et al., 2019; Sulaiman et al., 2017). However, no previous reports were found regarding the interaction between A. clavatus and NO neither in the field nor in vitro. Besides, Aspergillus spp. are the most commonly encountered fungi associated with allergy and pulmonary respiratory diseases, including A. fumigatus, A. niger, A. oryzae, A. flavus, and A. terreus. Therefore, in a context relating to FeNO levels, A. fumigatus was implicated as it is phylogenetically close to the A. clavatus (Krimizas et al., 2013; Samson et al., 2014). A study conducted by Sullivan et al. (2020) found that the T2 cytokines, IL-17, and TNF-α were positively correlated with A. fumigatus present in the bronchoalveolar lavage (BAL) of asthmatic patients. Consequently, upregulation of these cytokines induced the expression of iNOS which led to the prolonged release of NO. A similar finding was observed in a recent study conducted by Vandenborgh et al. (2020).

The analysis also discovered that the relative abundance of C. parapsilosis was positively associated with FeNO levels among asthmatic school children. However, no previous study has assessed this relationship. Hence, this finding was further explained by alluding C. parapsilosis to C. albicans as they formed cohesive clades of phylogenetic analysis (Diezmann et al., 2004). One previous study reported that C. parapsilosis infections account for the second-largest proportion of invasive candida infections after C. albicans among intensive care units (ICUs) patients throughout China (Gong et al., 2016). Several studies indicated that the β-glucan from C. albicans induces neutrophilic airway inflammation and expression of IL-17 and IL-5 (Inoue et al., 2009; Mori et al., 2001; Ramirez-velazquez et al., 2013). The effects of IL-17 on iNOS expression are modulated by various cytokines and follow complex pathways. Mikovic and Trajkovic (2004) suggested that binding of IL-17 to its receptors results in activation of MAPKs, NF-κb and expression of IRF-1, which in turn promote iNOS transcription. However, recent evidence indicates that the IL-5 pathway is not responsive to modulating the NO among asthma patients (Fuschillo et al., 2020).

S. melinoninorum, V. prosopidis and T. pseudoveloxum are commonly isolated from plants, insects and soil sources but host preferences of their respective genus are still largely not documented (Bezerra et al., 2017; Gonçalves et al., 2019; Santos et al., 2018). Moreover, very limited information on their medical importance is available for providing a comprehensive explanation of the association with FeNO levels. Additionally, due to the complexity of airway inflammation responses to different air pollutants, the aforementioned associations need to be interpreted carefully.

Our study has several limitations. Firstly, it is possible that we may have underestimated the diversity and the relative abundance of fungi in the settled dust samples due to the loss of fungal cells during the centrifugation, vortexing or enzymatic lysis steps in the DNA extraction procedures (Mbareche et al., 2019). Therefore, to obtain the greatest potential fungal
cell recovery, the newly designed extraction technique should be utilised. Secondly, the possibility of chimeras generation in the bioinformatics process might lead to errors in diversity interpretation (Cuadros-Orellana et al., 2013). Thirdly, we did not establish the relationship between FeNO levels and the school children’s personal characteristics. In future research, we would like to focus on personal traits, long-term observations and conducting multiple sampling events for dust samples. Another shortcoming is regarding the investigation of the dose-response effects between air pollutants and FeNO levels, which was impossible due to a lack of personal exposure samples and short-sampling frames. Another limitation to this study is that the temporal variability of exposure time and measurement taken may limit the ability to determine the potential effect on respiratory outcomes related to the pollutants exposure (Gaffin et al., 2018). Finally, through the nature of the study design, the cross-sectional study design utilised here restricts causal inference.

5. Conclusions

In summary, the findings from this detailed exploratory analysis revealed that the relative abundance of A. clavatus, B. acaciae, C. parapsilosis, H. does, H. multicystidum, H. shearii, S. mellinoporum, V. prosopidis and concentration of PM2.5 were found to be a significant predictor of increased FeNO levels among doctor-diagnosed asthma group. Therefore, advances in probing techniques to characterise the fungi are driving discovery in the relationship between rare and unculturable indoor fungi and biomarkers of airway inflammation. Moreover, these findings provide insights for future studies, aiming for a better understanding of long term airway inflammatory response and accounting the variability of exposure patterns (geographical, seasonal and latitudes).

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CRediT authorship contribution statement

Khalirul Nizam Mohd Isa: Software, Formal analysis, Investigation, Data curation, Writing for a better understanding of long term airway inflammation among doctor-diagnosed asthma group. Therefore, advances in probing techniques to characterise the fungi are driving discovery in the relationship between rare and unculturable indoor fungi and biomarkers of airway inflammation. Moreover, these findings provide insights for future studies, aiming for a better understanding of long term airway inflammatory response and accounting the variability of exposure patterns (geographical, seasonal and latitudes).

Khairul Nizam Mohd Isa: Conceptualization, Methodology, Resources, Supervision.

Jamal Hisham Hashim: Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition.

Lung Than: Conceptualization, Methodology, Resources, Supervision.

Juliana Jalaludin: Data curation, Writing for a better understanding of long term airway inflammation among doctor-diagnosed asthma group. Therefore, advances in probing techniques to characterise the fungi are driving discovery in the relationship between rare and unculturable indoor fungi and biomarkers of airway inflammation. Moreover, these findings provide insights for future studies, aiming for a better understanding of long term airway inflammatory response and accounting the variability of exposure patterns (geographical, seasonal and latitudes).


Declaration of competing interest

The authors declare that they have no conflicts of interest.

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